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Adult rat retinal ganglion cells and glia can be printed by piezoelectric inkjet printing

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Abstract

We have investigated whether inkjet printing technology can be extended to print cells of the adult rat central nervous system (CNS), retinal ganglion cells (RGC) and glia, and the effects on survival and growth of these cells in culture, which is an important step in the development of tissue grafts for regenerative medicine, and may aid in the cure of blindness. We observed that RGC and glia can be successfully printed using a piezoelectric printer. Whilst inkjet printing reduced the cell population due to sedimentation within the printing system, imaging of the printhead nozzle, which is the area where the cells experience the greatest shear stress and rate, confirmed that there was no evidence of destruction or even significant distortion of the cells during jet ejection and drop formation. Importantly, the viability of the cells was not affected by the printing process. When we cultured the same number of printed and non-printed RGC/glia cells, there was no significant difference in cell survival and RGC neurite outgrowth. In addition, use of a glial substrate significantly increased RGC neurite outgrowth, and this effect was retained when the cells had been printed. In conclusion, printing of RGC and glia using a piezoelectric printhead does not adversely affect viability and survival/growth of the cells in culture. Importantly, printed glial cells retain their growth-promoting properties when used as a substrate, opening new avenues for printed CNS grafts in regenerative medicine.

(Some figures may appear in colour only in the online journal)

Introduction

Inkjet printing of cells is an emerging technology to create cell-based structures essential in many regenerative medicine applications. There is great need in several neurodegenerative diseases and injuries of the brain and the spinal cord to find alternative methods to replace degenerated and injured cells

and provide growth-promoting substrates for regenerating cells in order to promote functional recovery.

In most inkjet printers the mechanical impulse that ejects the liquid drops is provided either by the expansion of a thermally-generated vapour bubble (thermal inkjet) or by the movement of a piezoelectric ceramic element (piezoelectric inkjet). Both methods have been shown to be useable for printing live cells. Several studies have shown that the heat and mechanical stress generated in thermal inkjet printheads only minimally affects viability of several cell types including cell lines, hamster ovary cells, muscle and stem cells [1–3]. Piezoelectric printers are less commonly used; it has been suggested that the specific vibration frequencies and power levels used may disrupt cell membranes and cause cell death

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[1, 4]. So far only embryonic neuronal cell types, including hippocampal, cortical and motor neurons have been tested for their viability after printing with thermal inkjet printers [1, 2]. We are not aware of any studies where inkjet technology has been used successfully to print viable cells derived from the eye, or any other part of the mature adult central nervous system (CNS), which is an important step in the development of tissue grafts for regenerative medicine, and may aid in the cure of blindness. In contrast to embryonic cells, adult neuronal cells of the CNS have limited ability to survive and regenerate [5] and therefore we hypothesized that they might be more prone to be affected by the printing process, in particular using a piezoelectric printhead.

In the current study we therefore tested the effects of inkjet printing, using a piezoelectric printhead, on two types of adult rat CNS cells—retinal ganglion cell (RGC) neurons and retinal glia. We initially assessed viability of printed dissociated retinal cells, of which RGC make up a small proportion [6] and of printed purified retinal glia, and subsequently assessed survival and regeneration-promoting properties of these cells in culture to see if these were affected by the printing process.

Materials and methods

Ethics statement

Animals had unrestricted access to food and water, and were maintained on a 12 h light/dark cycle. Animal work was conducted in accordance with the UK Home Office regulations for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act (1986) and the ARVO statement for the use of animals in Ophthalmic and Vision Research. All methods were approved by the University of Cambridge Animal Ethics Committee and are in conformity with the 'Guiding Principles for Research Involving Animals and Human Beings' as adopted by the American Physiological Society. Animals were humanely sacrificed by exposure to CO₂.

Inkjet printing and imaging apparatus

A single nozzle piezoelectric inkjet device (MicroFab, Texas, USA) was used to print purified retinal glial and dissociated retinal cells. Consisting of a glass capillary tapered to a sub-millimetre diameter nozzle and surrounded by a piezoelectric actuator, the printhead ejects fluid drops when a specific electrical pulse is supplied. The driving waveform was defined by a PC-driven generator (Jet Drive II, MicroFab). For the cell jetting experiments, an asymmetric bipolar waveform repeating at 1 kHz frequency was used with pulse magnitudes between 50 and 80 V, tailored to the nozzle size used. The retinal and glial cells were jetted from devices with 50 and 80 μ m diameter nozzles, respectively. About 8 cm of C-FLEX flexible thermoplastic tubing connected the print device to the 3 ml fluid reservoir holding the cell suspension. A custom-built pneumatic/vacuum controller maintained a slight negative pressure in the fluid reservoir to control the nozzle meniscus level for optimal jetting. Prior to jetting, the printing device

was cleaned in diluted alkaline cleaning solution (Micro-90, Cole-Parmer, USA) and back-flushed with isopropanol and sterilized water to ensure that no residue remained. Immediately before cell jetting, the print device, the fluid reservoir, and the supply tubing were flushed first with cell specific medium (described below) before being filled with cell suspension. The cells were printed with a flight distance of approximately 10 mm directly into a vial.

A high-speed video system [7] was used to monitor the cell printing process and to study the distortion experienced by the cells during printing. The nozzle region was back-illuminated with a high-intensity flash source (Specialised Imaging SIMSFH-500, UK; flash energy 500 J, duration 2 ms), and an ultra high-speed camera (Shimadzu HPV-1, Japan) was used to record 102-frame videos of cell jetting at 0.5 million frames per second. The cell jetting apparatus and imaging arrangement is shown schematically in figure 1(A).

Tissue culture

Glial cultures. Retinal tissue was derived from adult male Sprague Dawley rats (Charles River, Margate, UK).

Retinal glial cultures were prepared as described previously [8–10]. For this, one day after plating, nonattached neuronal cells were removed by gentle agitation and change of the medium, Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (Invitrogen, Paisley, UK). The medium was subsequently changed every 2–3 days, resulting in highly purified glial cultures, containing astrocytes and Müller glia, after two weeks of culture as described previously [8–10]. To prepare cells for printing, cells were washed with calcium/magnesium-free phosphate buffered saline (PBS) (Invitrogen), incubated for 5 min in a 1 x Trypsin-EDTA solution (Invitrogen), shaken to detach the cells, and medium was added. After centrifugation and removal of supernatant, cells were resuspended in DMEM (and 10% fetal calf serum) and passed through a cell strainer (BD, Oxford, UK) to remove cell clumps. Half of the cell suspension was used for inkjet printing, with the other half serving as a control. After inkjet printing the cells into a new vial, viability and number of live cells were assessed in both printed and control conditions, determined by the trypan blue test.

Equal volumes of the two cell suspensions were plated in DMEM (and 10% fetal calf serum) on 13 mm diameter coverslips (VWR, Lutterworth, UK) in Nunc 4 well plates (Fisher Scientific, Loughborough, UK) at a number of approximately 9000 glial cells for the control cells. Coverslips were pre-coated with 100 μ g ml⁻¹ poly-L-lysine (Sigma, Poole, UK). Medium was changed every 2–3 days. Cells were kept at 37 °C in a humidified atmosphere containing 5% CO₂. After one week, prior to plating the retinal cells, the medium was changed three times to B27 supplemented Neurobasal-A medium (Invitrogen).

Retinal cultures. To prepare dissociated retinal cultures from adult male Sprague Dawley rats, a papain dissociation kit (Worthington Biochemicals, New Jersey, USA) was used according to the manufacturer's instructions. Dissociated

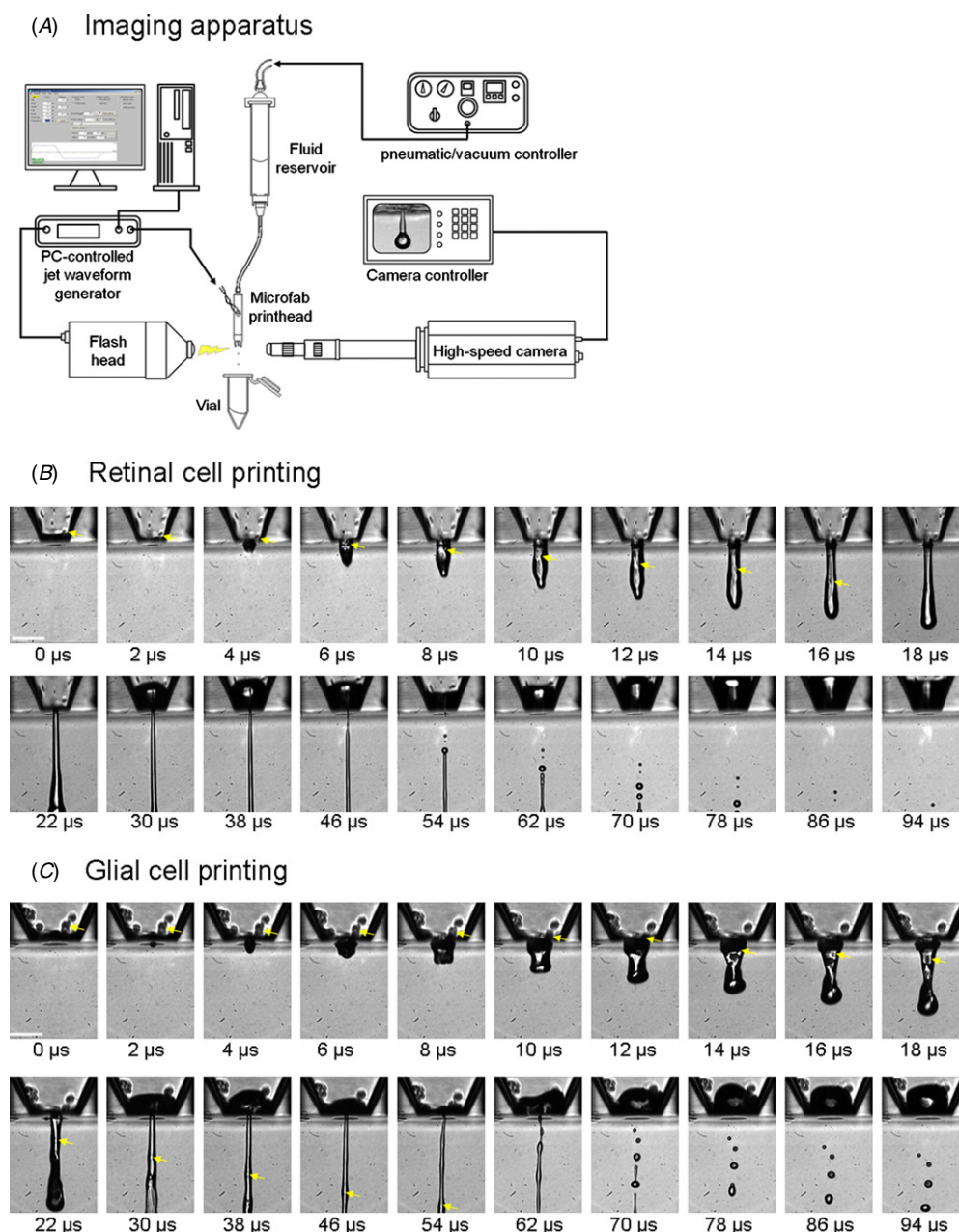


Figure 1. (A) Schematic of the inkjet printing and imaging apparatus used to study printing of purified retinal glial and dissociated retinal cells. Image sequences of (B) retinal cells and (C) purified glial cells as they are ejected from the nozzle, labelled with image capture times. Close-up images of (D) retinal cells and (E) glial cells in jets. (F) Snapshots of settled retinal cells in nozzle during jetting. Scale bar: 100 μ m. The arrows indicate individual cells tracked for analysis. (Continued on page 4.)

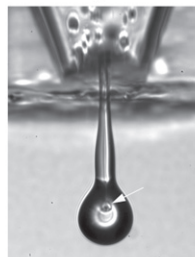
retinal cells were resuspended in B27 supplemented Neurobasal-A medium and passed through a cell strainer to remove cell clumps. Half of the cell suspension was used for inkjet printing, with the other half serving as a control. After inkjet printing the cells into a new vial, viability and number of live cells were assessed in both printed and control conditions, determined by the trypan blue test. Equal volumes of the two cell suspensions were plated in Nunc 4 well plates on poly-L-lysine coated 13 mm diameter coverslips or on the glial cells prepared above, at a number of approximately 65 000 retinal cells for the control cells. Cells were cultured for a further five days at 37 °C in a humidified atmosphere containing 5% CO₂.

Three wells were plated per experimental condition and each experiment was repeated three times. Since we found the number of printed cells to be lower compared to controls after inkjet printing (table 1), in two of the experiments we plated control cells at the same number as the printed cells, as additional conditions.

Immunocytochemistry

Cells were fixed with 4% PFA at room temperature for 10 min and stained for β III-tubulin and Vimentin as previously described [10]. β III-tubulin is a phenotypic marker for RGC somata and their processes [11–13] and Vimentin is a marker

(D) Close-up of retinal cells in a jet



(E) Close-up of glial cells in a jet



(F) Retinal cell settlement in nozzle during jetting

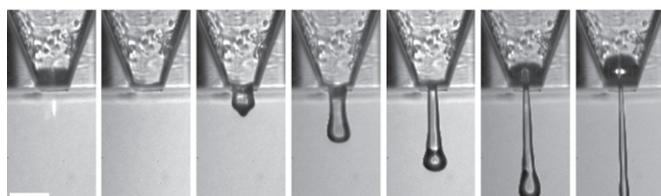


Figure 1. (Continued.)

for retinal glia, astrocytes and Müller glia [14]. After blocking in 3% BSA and 0.1% Triton in PBS, primary antibodies, rabbit anti- β III tubulin (1:1000, Covance, Cambridge Bioscience, Cambridge, UK) and mouse anti-Vimentin (1:500, Sigma), were applied in blocking solution overnight at 4 °C. The following day coverslips were washed in PBS and secondary antibodies were applied for 1 h at room temperature (1:1000 in blocking solution; Alexa Fluor 555 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG, Invitrogen). Coverslips were washed in PBS, followed by nuclear counterstaining with 4',6-diamidino-2-phenylindole (1:10 000, DAPI, Sigma). After a final wash in PBS, coverslips were mounted in Fluorsave.

Images of 30 randomly selected RGC per experimental condition were captured, using a standard epifluorescence microscope (model DM6000B; Leica, Wetzlar, Germany), and the length of their longest neurite was measured using the

Leica Application Suite (LAS AF.1.8.0) program. In addition, β III tubulin+ RGC, as well as Vimentin+ glia (defined by their nuclei) were counted in nine same-sized areas/well, averaged and the total number of β III tubulin+ RGC and Vimentin+ glia/well estimated, as previously described [10]. Results were averaged and expressed as mean \pm standard error of the mean (SEM). The differences between means were evaluated by an unpaired two-tailed *t* test (assuming equal variances) and considered significant at $P < 0.05$ ($P < 0.05 = *$; $P < 0.01 = **$; $P < 0.001 = ***$).

Results

Mechanics of the printing process

Typical image sequences showing dissociated retinal cells and glial cells being jetted from the transparent glass nozzles are shown in figures 1(B) and (C), respectively. Two different nozzle sizes (50 and 80 μ m in diameter) were used to account for the difference in size between the retinal and glial cells, in order to minimise nozzle clogging. The average maximum jet velocity from the 50 μ m nozzle (retinal cells) is around 13 m s⁻¹, compared with around 10 m s⁻¹ from the 80 μ m nozzle (glia). In both cases the ejected droplets show characteristic long ligaments trailing the main drops which broke up into small satellite drops at later times; these are typical of the jets formed by Newtonian liquids without the presence of cells. Although no rheological measurements were made, the general similarity between the jetting behaviour of the cell suspensions and that of other Newtonian fluids suggested that any non-Newtonian effects such as shear thinning/thickening or viscoelasticity were insignificant under our jetting conditions.

High shear rate (and hence shear stress) are nevertheless present in the fluid during the jetting process. The shear and extensional forces imposed on the jetted cells are likely to be highest during the early stage of ejection as they pass through the nozzle. Maximum fluid shear rates may be estimated from the ratio of the jet speed to the nozzle diameter to be of the order of 10⁵ s⁻¹. It took from 2–5 μ s for the jets to reach their maximum velocity, corresponding to an average acceleration of at least 10⁶ m s⁻² (or 10⁵ times that of gravity). Observations of the cell motion when a jet was fully formed showed that the acceleration in the later stages was significantly lower. Despite the very high shear rate and acceleration to which the cells had been subjected, real-time observation of the glass nozzle during jetting showed no sign of active cell disintegration. In

Table 1. Effects of inkjet printing on cell number and viability, and subsequent number of plated cells. Cells (retinal glia, dissociated retinal cells) were derived from adult rats. Control = non-printed cells. Results consist of the means (\pm SEM) of three separate experiments.

	Control	Printed
Viability of glial cells	78.4 \pm 8.2%	69 \pm 12.2%
Number of live glial cells/ml	134 000 \pm 18 000	57 000 \pm 18 000
Viability of retinal cells	74.3 \pm 2.6%	69 \pm 5.3%
Number of live retinal cells/ml	778 000 \pm 19 000	520 000 \pm 29 000
Number of plated glial cells/well	9400 \pm 750	3800 \pm 560
Number of plated retinal cells/well	67 000 \pm 4000	45 000 \pm 400

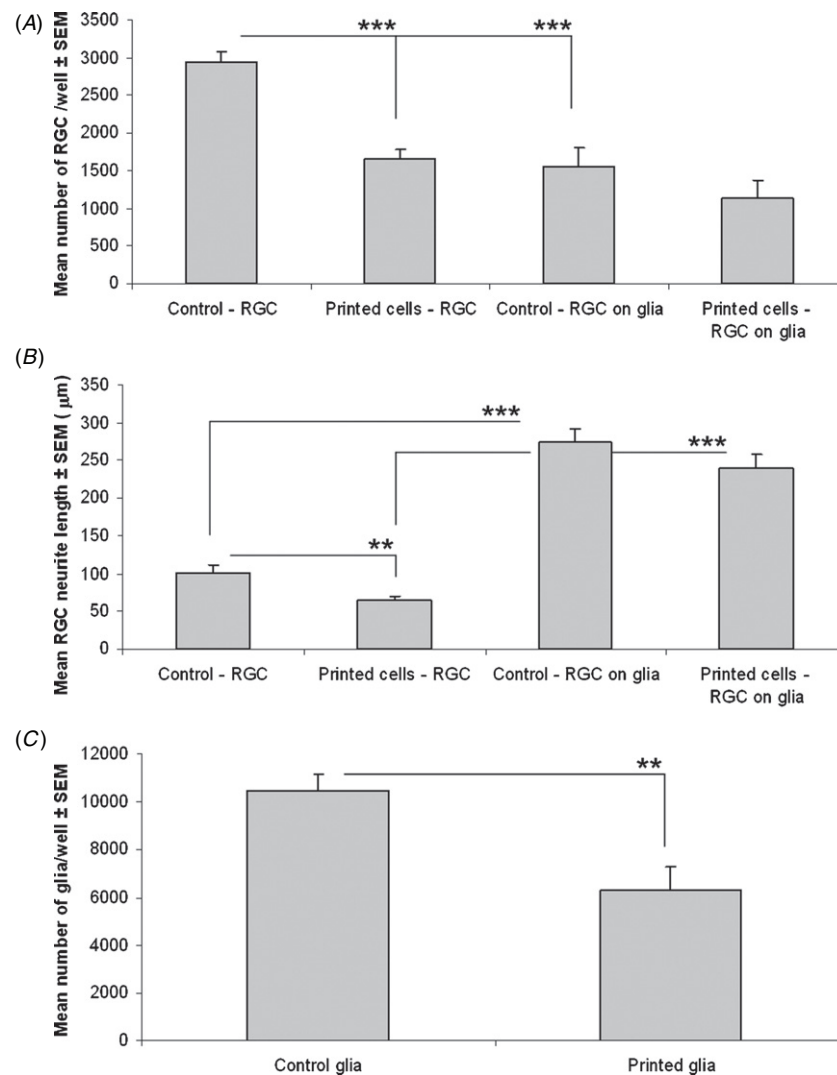


Figure 2. The printing process appears to affect RGC/glial numbers and RGC neurite outgrowth. (A) Number of RGC surviving in culture, (B) mean RGC neurite lengths (in μm), and (C) number of retinal glia surviving in culture. Cells were derived from adult rats and had either undergone a printing process or not (control). Retinal cells were either plated on their own or on a glial substrate. Results consist of the means (\pm SEM) of three separate experiments. Significant differences in A and B are indicated by asterisks ($P < 0.01 = **$; $P < 0.001 = ***$).

addition, close-up images taken during the initial jetting phase (50 μm nozzle/retinal cells, figure 1(D); 80 μm nozzle/glial cells, figure 1(E)) using an automated ultra-fast freeze-frame imaging arrangement as previously described [15] show no evidence of significant deformation once the cells are outside the printhead.

Effects of the printing process on cell number and viability

We assessed the effects of the printing process on cell number and viability before plating the cells. We found that printing led to a reduction in cell numbers of both retinal glial cells and dissociated retinal cells. On average there were around 57% fewer glial cells and 33% fewer retinal cells present in the printed samples compared to the non-printed controls. Post-printing inspections of the printhead revealed cells attached to the inside of the glass capillary. In addition, freeze-frame images of the nozzle area after a prolonged period of jetting, as shown in figure 1(F), showed retinal cells settling just above the meniscus fluctuation zone. Associating these observations

with the evidence of minimal cell deformation during jetting, we believe that cell sedimentation, rather than cell destruction, is the likely cause of the reduction in the final cell counts. Crucially, the viability of the cells was not significantly affected by the printing process (table 1).

Effects of the printing process on RGC/glial survival and RGC neurite outgrowth in culture

We next wanted to determine whether the survival and growth of the cells in culture was affected by the printing process.

When we cultured retinal cells by themselves, we initially found a significant reduction in the number of printed RGC after the 5 day culture period compared to control ($P < 0.001$; figures 2(A) and 4(A), (B)). Furthermore, there appeared to be a small but significant reduction in neurite outgrowth from printed RGC compared to control ($P < 0.01$; figures 2(B) and 4(A), (B)). However, when we plated matching numbers of unprinted (control) and printed RGC, we found no significant

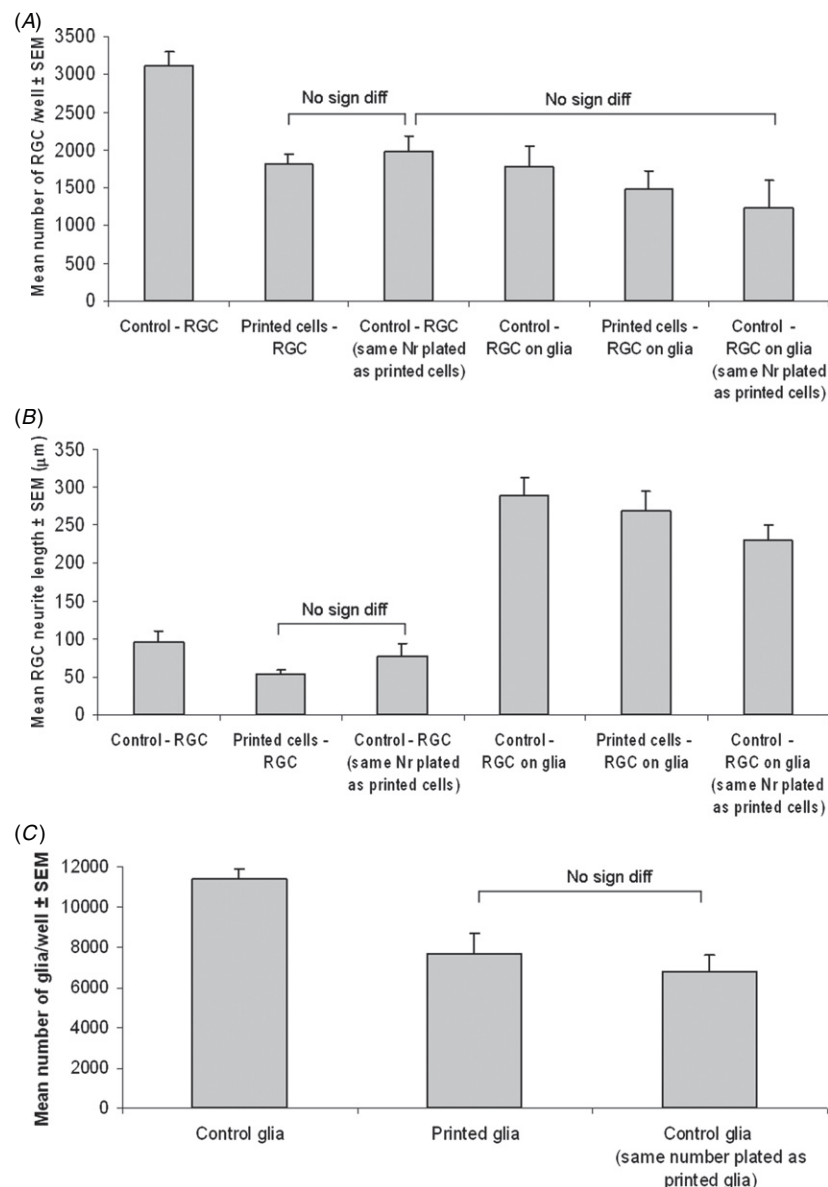


Figure 3. Adjustment for the reduction in cell number that occurred during the printing process shows that printed RGC/glia do not differ in culture from non-printed control cells. (A) Number of RGC surviving in culture, (B) mean RGC neurite lengths (in μm), and (C) number of retinal glia surviving in culture. Retinal cells were either plated on their own or on a glial substrate. Retinal cells and glia were also plated at the same number as their printed counterpart. Results consist of the means (\pm SEM) of two separate experiments.

difference in survival and neurite outgrowth between control and printed RGC (figures 3(A), (B) and 4(B), (C)).

Similarly, when assessing the number of printed glia present after the culture period, we initially found a significant reduction compared to control (figures 2(C) and 4(J), (K)). However, when we plated matching numbers of unprinted (control) and printed cells, we found no significant difference (figures 3(C) and 4(K), (L)).

Hence, the printing process does not appear to have adversely affected the survival/regeneration properties of these cells in culture.

Effects of a glial substrate on RGC survival and neurite outgrowth

We found that RGC grew significantly longer neurites when plated on a glial substrate ($P < 0.001$; figures 2(B) and 4(A),

(D), (M), (P)). This occurred to a similar extent when the glial cells and RGC had undergone a printing process prior to plating ($P < 0.001$; figures 2(B) and 4(B), (E), (N), (Q)). When assessing the number of RGC present after the culture period we found that in controls, there were significantly fewer RGC present when cultured on a glial substrate ($P < 0.001$; figures 2(A) and 4(A), (D), (M), (P)), whilst there was no difference when both cell types had been printed (figures 2(A) and 4(B), (E), (N), (Q)). However, we found that untreated glial cells, when plated at the same number as the printed glial cells, did not significantly affect RGC survival (figures 3(A) and 4(C), (F), (O), (R)).

Hence, glial cells that have undergone a printing process retain their growth promoting properties when used as a substrate.

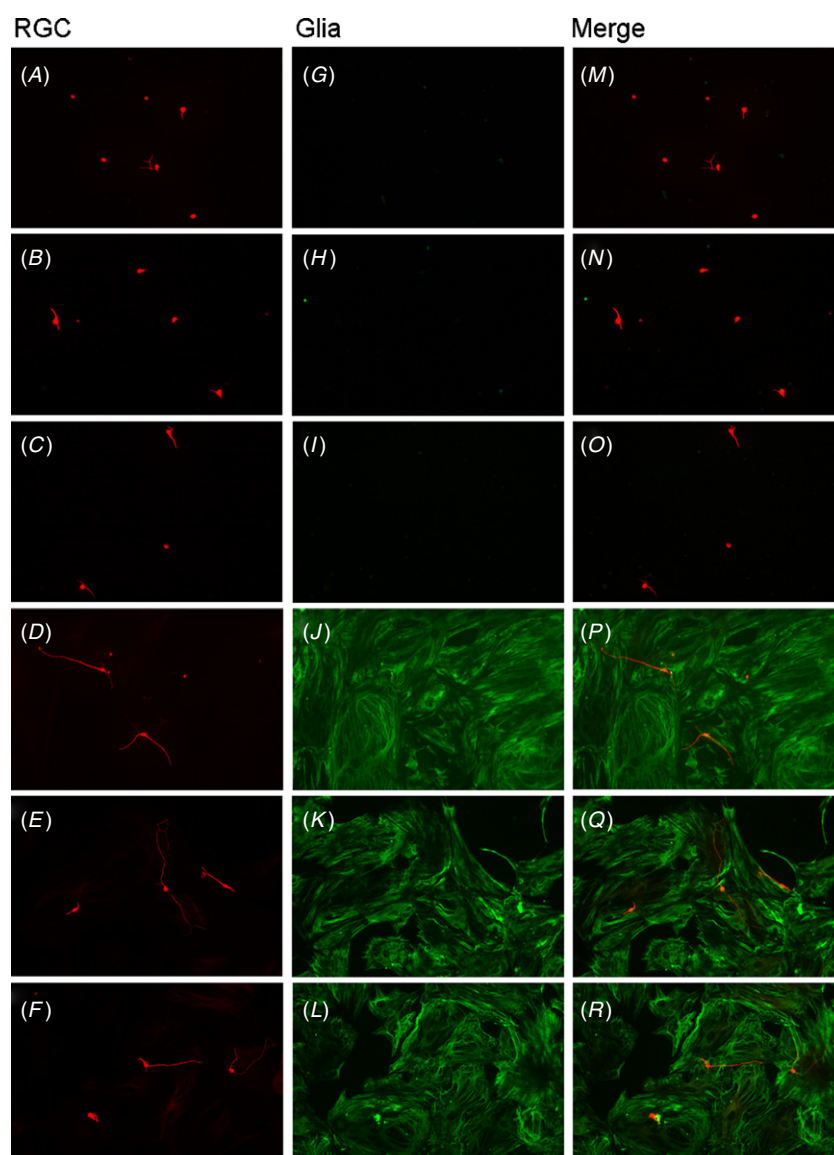


Figure 4. Photomicrographs of β III tubulin+ RGC (red) and Vimentin+ glia (green) in cultures from (A),(D) control retinal cells, (B),(E) printed retinal cells and (C),(F) control retinal cells plated at the same number as the printed retinal cells, either on their own (G)–(I) or with the retinal cells additionally having been plated on (J) control glia, (K) printed glia or (L) control glia plated at the same number as the printed glia. Scale bar: 50 μ m.

Discussion

It has previously been shown that inkjet printing can be used to deposit mammalian cells in a defined pattern and to create cellular structures. So far studies have been focused on printing cell lines, embryonic neurons or other cells including muscle cells. It was found that viability was minimally affected and the normal phenotype of the printed cells was retained [1–3, 16]. In the present study we tested whether cells from the adult CNS, RGC and glia, would be affected by inkjet printing with a piezoelectric printhead.

A concern raised by some previous investigators was that the vibration frequency in piezoelectric printheads may lead to cell membrane disruption and cell death [1, 4], and most previous cell printing studies were performed with thermal inkjet printers [1–4, 17]. It has however been shown that piezoelectric printers can be modified to print insect cells,

endothelial cells and fibroblasts, without affecting cell viability [16, 18, 19].

Using the single nozzle MicroFab printhead as an analogue to typical commercial, multi-nozzle printheads, we were able to image and study cell jetting dynamics in real-time. We have shown that a piezoelectric printhead operating at drop repetition rates up to 1 kHz with drop ejection speeds up to 13 m s^{−1} does not significantly affect the viability of printed adult RGC and glial cells. Although the cells are subjected to very high shear rates and acceleration during jetting, no significant distortion of the cell structures has been observed either immediately before or after cell ejection. The observations suggest that either the cell membranes possess sufficient strength and elasticity to resist a brief period of high stress, or the geometry of the printhead nozzle used results in rather little shear or deformation of the cells during jetting. Further studies in this area will hopefully develop a better

understanding of the dynamics of cell-fluid flow interaction during printing.

The concentration of cells in the medium passing through the printhead was found to be significantly lower than that present in the original suspension. On average there were 57% fewer glial cells and 33% fewer RGC retained compared with the non-printed control. Post-jetting examinations of the printhead revealed a large number of cells adhering to the glass capillary wall near nozzles, suggesting that the tendency of cells to settle and adhere to the internal surfaces of the feeding tube and printhead may be a primary mechanism for the cell loss experienced. This is a commonly reported problem for piezoelectric inkjet printers that can be overcome by using various modifications which we will test in future for compatibility with our printing system and cells [16, 19–22].

RGC survival and neurite outgrowth after the 5 day culture period appeared to be significantly reduced in printed cells compared to control. Experiments were repeated three times. However, when we plated in two of the experiments untreated retinal cells at the same number as the printed cells, we found no significant difference in survival and neurite outgrowth. A possible explanation for the reduction in neurite outgrowth that we observed initially is that the RGC themselves release growth factors as brain derived neurotrophic factor which has been shown to promote neurite outgrowth [23, 24], and that consequently neurite outgrowth may be affected by the number of RGC present in culture. Overall these results suggest that RGC survival and neurite outgrowth are not affected by the printing process.

We and others have previously shown that retinal glia, astrocytes and Müller glia, when activated following injury, release growth factors such as ciliary neurotrophic factor, leukemia inhibitory factor and Apolipoprotein E, that underlie successful regeneration of RGC axon growth past the optic nerve lesion site *in vivo* and enhanced RGC neurite growth in culture [25–27]. We show here that retinal glia from untreated adult rats are also able to highly significantly ($P < 0.001$) promote RGC neurite outgrowth when used as a substrate. This may be mediated through growth factors such as brain-derived neurotrophic factor and basic fibroblast growth factor which Müller glia from untreated rats have been shown to express [28, 29]. Importantly, we found that growth of printed RGC on a substrate of printed glia was similarly enhanced ($P < 0.001$) as we had observed for the unprinted control cells, suggesting that printed glia retain their growth promoting properties and printed RGC their phenotype which allows them to respond to these factors.

Conversely, we found that RGC survival on the glial substrate was significantly reduced in control. This did not occur in the printed cells, or when we plated control glia at the same number as the printed glia. This suggests that at high density glial cells may produce factors that are detrimental to RGC attachment or survival.

The fact that RGC and retinal glial cells do not appear to be affected by the printing process and retain their phenotype in culture opens up the possibility for studying interactions of these cells when printed in precise locations and patterns. This could enable the creation of cell arrays mirroring the

in vivo situation, which would allow screening the effect of novel compounds on cell–cell interactions before application *in vivo*.

It also opens new avenues for creating printed tissue grafts for use after CNS injury *in vivo*. It has been shown previously that inkjet printing can be used to transfect cells by co-printing plasmids encoding green fluorescent protein together with porcine aortic endothelial or Chinese hamster ovary cells, which resulted in a transfection efficiency of 10 and 30% respectively [4, 17]. In addition it was shown that it was possible to print a three dimensional fibrin gel scaffold which included layers of transfected porcine aortic endothelial cells that were viable *in vivo* and started expressing green fluorescent protein. This ‘side effect’ of the printing process which utilizes transient pore opening in the membranes of the printed cells, therefore opens the possibility of simultaneous transfection and delivery of cells into tissue constructs for implantation *in vivo* [17]. So far this has only been demonstrated with a few cell types, using thermal inkjet printers. It will be interesting to investigate in future whether RGC and glia can be transfected in a similar way using piezoelectric inkjet printers. Furthermore, with the established use of fibrin *in vivo* [30, 31], and the growth promoting effects of a substrate of printed glial cells, as observed in the present study, it will be interesting to investigate in future studies if a printed fibrin-glia construct might promote functional recovery following optic nerve or spinal cord injury *in vivo*.

It will furthermore be important to extend this study to other cells of the retina and to investigate if light-sensitive photoreceptors can be successfully printed using inkjet technology. If this can be proven, printing of a functional retina for the cure of some forms of blindness could be within reach.

To achieve these future aims, the findings of the present study, which used a single nozzle system, will need to be translated to commercial, multi-nozzle printheads, as have been used in other work [21]. It is known that a MicroFab printhead can eject fluids with a greater viscosity range than typical commercial printheads, and its tapered glass capillary nozzle may induce less shear stress on the jetted cells. Therefore, additional efforts may be needed to tailor the cell suspension rheology and jetting parameters such as drive waveform for use in a commercial multi-nozzle printing system. Furthermore, modifications to reduce cell sedimentation in the printhead [19–22] will need to be implemented, to allow printing of cells over a prolonged period, without loss of yield.

Conclusions

This study is the first to show that cells of the adult CNS (RGC and glia) can be printed using a piezoelectric printer without adverse effects on viability of the printed cells. RGC/glia survival and RGC neurite outgrowth in culture did not appear to be affected by the printing process itself. Importantly, we found that printed glial cells retain their growth promoting properties which opens the possibility for developing printed grafts for use in regenerative medicine.

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